

Classification and Characterization of Ten Hemocytes Types in the Tunicate *Halocynthia roretzi*

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ABSTRACT—We propose a standard condition for classifying living hemocytes of *Halocynthia roretzi*: adhering or spreading on glass in the presence of EGTA at pH 6.0 at room temperature (adhering cells). According to morphological characteristics revealed by vital staining, autonomous fluorescence and an effect of NH₄ ions, we classified hemocytes into ten groups; phagocytes type 1 and 2 (p1- and p2-cells), granular cells type 1, 2, and 3 (g1-, g2- and g3-cells), vacuolated cells type 1, 2, 3 and 4 (v1-, v2-, v3-, and v4-cells) and lymphoid cells (ly-cells). Two types of phagocytes, p1- and p2-cells, were the only cells which actively phagocytize and spread as adhering cells. They were spherical cells with a diameter of 20–40 μ m and 40–50 μ m before spreading. Three types of small-granule containing cells and four types of large-vacuole containing cells were also clearly distinguished not only by their size but also by their behavior or properties after vital staining. Ly-cells were small and spherical and have been referred to as lymphocytes or hemoblasts. We correlated seven groups (p1-, p2-, g1-, g2-, v3-, v4- and ly-cells) with the former classification, and identified for the first time three types of hemocytes (g3-, v1- and v2-cells). The relative proportions of various hemocyte types did not agree with earlier reports, and p1-cells, accounting for 30–45%, were found to be the largest group. These basic analyses of cell types are essential for future studies of immunodefense properties.

INTRODUCTION

The tunicate *Halocynthia roretzi*, is an excellent protochordate for research dealing with hemocytes and hemolymph. Each individual is large and therefore it contains sufficient quantities of hemolymph and the animals can be supplied throughout the year. Consequently, it is one of the tunicates whose hemolymph has been well investigated biochemically [1–3]. With respect to the cells, their hemocytes, there is the earlier classification by Ohuye [4] and a later detailed one by Fuke [5]. Turning to function, alloreactivity of hemocytes has also been studied by Fuke [6, 7] and is well known as the “contact reaction”. However, these results have not sufficiently established the classification of hemocytes and it is important to identify and classify them according to their func-

tions.

For the purpose of investigating their functions, one approach to identifying individual hemocytes in the living state must be established. However, hemocytes easily aggregate which makes it difficult to identify individual cells. In addition, the morphological characteristics of hemocytes undergo substantial change after they adhere on a matrix, after degranulation, or during amoeboid movement. The fixation, staining methods and electron microscopy also cause deformation. Because of this instability, previous classifications were based upon fixed hemocytes after staining or on hemocytes modified after degranulation, spreading on a matrix or phagocytosis.

We considered it essential to classify living hemocytes in order to examine their function. Therefore, we searched for: 1) the condition in which hemocytes degranulation was mostly suppressed; 2) when morphological differences among hemocytes became maximum. We were able to

identify each individual hemocyte separately in the living state. Finally we confirmed some of the studies by Fuke [5], and examined the differences between her reports and our observations.

MATERIALS AND METHODS

Hemocytes

Tunicates (*Halocynthia roretzi*) were collected at the Mutsu-bay in northeastern Japan, from April to October, and were transported immediately. Hemocytes were collected within 2 days (fresh-animals) and collected after maintenance in an aquarium for 4–8 weeks without feeding (4–8 weeks-animals). Hemocytes were collected from the space just beneath epithelium, at a tunic papilla in the upper part of the body, with 1 ml disposable plastic syringes. Harvesting was done carefully in the same way and at similar points from each individual. When we collected hemocytes in the presence of ethyleneglycol-bis(-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), 0.2 ml of the solution containing 5 mM EGTA and 0.5 M sodium chloride (EGTA-solution, pH 7.0 with HCl) was previously drawn into 1 ml plastic syringes and, with the same syringe, we collected 0.8 ml of hemolymph which included numerous hemocytes.

Behavior of hemocytes on glass slides at different pHs

Upon centrifuging the mixture of hemolymph and EGTA-solution at 250 g for 1 min, hemocytes were collected and resuspended in 1 ml of EGTA-solution. A small quantity (0–20 μ l) of 0.1 N sodium hydroxide was added to raise the pH of hemocyte suspensions to the range of pH 5.5–8.0. We then added about 100 μ l of the hemocyte suspension on a glass slide and the activity of hemocytes was viewed as they attached and spread. Phenol red (10–30 μ l of 0.1% solution in distilled water) was added to the hemocyte suspension to estimate the pH. Hemocytes were then observed by Nomarski-differential-interference microscopy (Nomarski-image) and by phase-contrast microscopy (phase-contrast-image).

Phagocytic activity for sheep red blood cell (SRBC)

Hemocytes in intact hemolymph were incubated with sheep red blood cells (SRBC) for 5 to 30 min. About 0.1% SRBC suspension (v/v) in normal sea water was added to the hemolymph, reaching the final concentration 0.005–0.01%.

Autonomous fluorescence

Hemocytes attaching or spreading on glass slides in EGTA-solution, in normal sea water or in intact hemolymph were examined under a fluorescence microscope with ultraviolet illumination.

Quantification of hemocytes

Hemocytes were collected in the presence of EGTA, rinsed and resuspended in EGTA-solution. After the pH of hemocyte suspensions was adjusted to pH 6.0, 0.1–0.2 ml was added to glass slides and incubated for 10–15 min. We identified all hemocytes within several different viewing areas (about 300 cells in total) by phase-contrast microscopy and counted the numbers of each hemocyte type. Hemocytes in the suspended state, in EGTA-solution at pH 5.5, were also counted.

Staining

Neutral red (0.01% in final concentration) was added to the hemocyte suspension and vitally stained hemocytes were observed after 15–30 min. Hemocytes adhering or spreading on glass slides in the EGTA-solution (pH 6.0) were fixed and stained with Wright's blood stain.

Effect of NH₄Cl or NH₄OH

NH₄Cl (5–500 mM) or NH₄OH (0.1–1%) was added to the hemocyte suspension in EGTA-solution or in intact hemolymph and examined by light microscopy 5–15 min later. Phenol red (0.01%) was added to the hemocyte suspension to estimate the pH.

RESULTS

Adhering and spreading abilities of hemocytes on glass slides

Hemocytes in intact hemolymph (collected without EGTA) tended to form small clusters containing 3–10 hemocytes. They aggregated more intensively after placing them on glass slides. In contrast, hemocytes in hemolymph containing EGTA (at pH 5.0–5.5) did not aggregate and thus each individual cell type could be observed (Fig. 1-A). The addition of EGTA-solution to hemolymph changed the pH to 5.0, from the original value of 6.0–6.5. No hemocytes adhered nor were observed to spread on glass slides under this condition.

Rinsed and resuspended in EGTA-solution at pH 5.0–5.5, hemocytes neither adhered nor spread on glass slides, but exhibited Brownian movement. Raising the pH caused increasing numbers of hemocytes to attach whereas some of them spread. In analyzing hemocytes from several fresh-animals, the percentage of spreading cells was low at pH 5.0–5.6 but it increased consider-

ably at pH 5.8–5.9. Although the pH-range and the sequential raising of the pH varied in three cases, the percentages of spreading cells reached a maximum in each of three cases at pH 6.0–6.1 (Fig. 2). Most of the spreading hemocytes under this condition were phagocytes type 1 and 2 according to the classification to be described later (Fig. 1-B). Above pH 6.5, the percentage of spreading cells tended to decrease slightly whereas other cell types spread at pH 7.0–8.0.

Temperature within the range of 15–25°C did not affect spreading and morphological characteristics of hemocytes, except that higher temperatures slightly accelerated their spreading. At 15°C, the number of spreading cells reached a plateau within at least 15 min. When hemocytes were placed on glass slides for 15 min in EGTA-solution at pH 6.0 at room temperature, all of phagocytes type 1 and 2 spread and all other hemocytes adhered revealing characteristic features for their

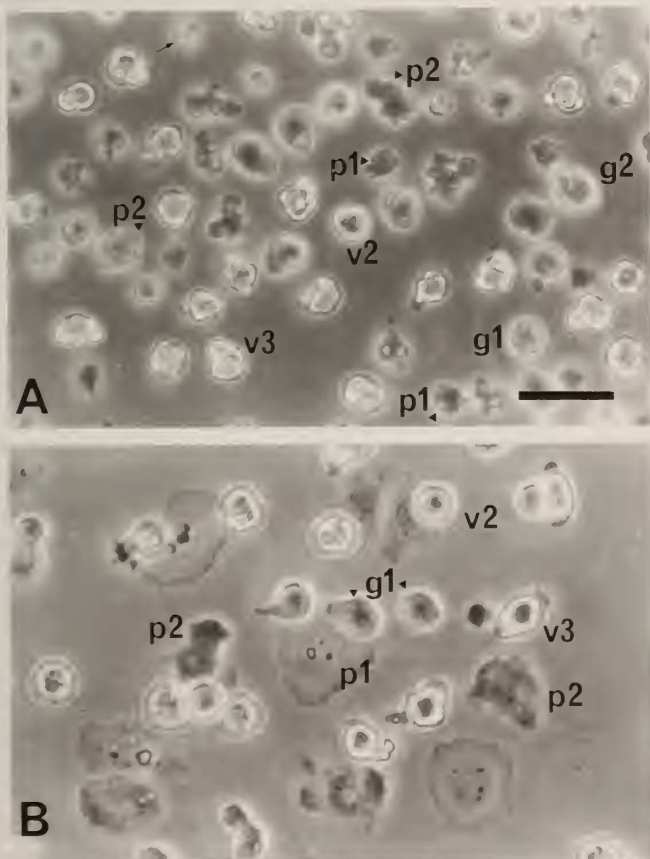


FIG. 1. The hemocytes of *Halocynthia roretzi* suspended in the EGTA-solution at pH 5.5 (A: the condition for cell suspension), and spreading or adhering on glass slides in the EGTA-solution at pH 6.0 (B: the condition for adhering cells). The scale bar shows 100 μ m. p1, p2: phagocyte type 1 and 2; g1, g2: granular cell type 1 and 2; v2, v3: vacuolated cell type 2 and 3.

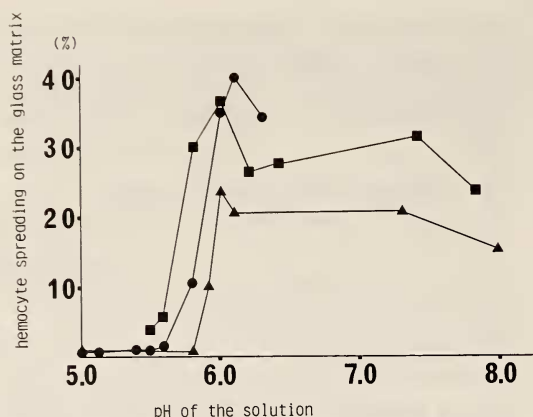


FIG. 2. The percentage of spreading hemocytes on glass slides at different pH. Square (■), triangle (▲) and circle (●) indicate the results in the hemocytes of three different individuals, respectively. Although three cases were examined at different pH and in the different pH range, it was considered that the spreading hemocytes reached a maximum at pH 6.0–6.1 in each case.

cell-types (Fig. 1-B). Afterward, we used this condition (the condition for adhering cells) as the standard to identify or classify living hemocytes: in the EGTA-solution at pH 6.0, placed on glass slides for 15 min at room temperature.

Classification of hemocytes at the condition for adhering cells

We classified hemocytes under the conditions for adhering cells into ten groups: two types of phagocytes, three types of granular cells, four types of vacuolated cells and lymphoid cells (Figs. 3, 4).

Phagocytes type 1 (p1-cells) were 80–100 μm in diameter and they spread over wide distances (Figs. 3-A, E; 5). They did not move or they moved so slowly that we only observed motion by intense observation. The Nomarski-image revealed that they spread as thin, flat sheets (Fig. 3-A). These sheets were transparent in the phase-contrast image (Figs. 3-E; 5-C, D) and usually contained several dark granules (Fig. 3-E). Phagocytes type 2 (p2-cells), 60–100 μm in diameter, were also spreading cells (Figs. 3-B, F; 6), modifying their shapes frequently by amoeboid movement: either rounding up, spreading extensively or sending out long pseudopodia. The Nomarski-

image revealed that they were thicker than p1-cells in the fully spread state (Fig. 6-E). They sometimes contained several granular components (Fig. 6-C, D, E). P2-cells were relatively dark in phase-contrast-image after they spread (Figs. 3-F; 6-C, D, E), even in a fully spread state.

Granular cells type 1 (g1-cells) 35–40 μm in diameter did not spread but retained the round shape (Fig. 4-A, E), adhering to glass slides by means of small pseudopodia; they sometimes moved actively. In the Nomarski-image they contained many small granules less than 5 μm in diameter (Fig. 4-A), and they were weakly refractile in phase-contrast-microscopy (Fig. 4-E). In a few cases, some of them expanded slightly into dark round cells. Granular cells type 2 (g2-cells) 45–50 μm in diameter were large, polygonal cells (Fig. 4-B, C, F, G). Rarely, did they have small protrusions. The Nomarski-image showed that these cells were filled with numerous small granules less than 5 μm in diameter (Fig. 4-B). In the phase-contrast image (Fig. 4-F), they were slightly brown and dark usually with a refractile periphery; these cells had no pseudopodia nor did they move. Granular cells type 3 (g3-cells) were large and oval, 80 μm in diameter (Fig. 4-D, H), the largest hemocyte; they did not move. Nomarski-image showed that these cells were filled with numerous small granules less than 5 μm in diameter (Fig. 4-D). They contained highly refractile granules in the phase-contrast-image.

Vacuolated cells type 1 (v1-cells) were small cells 30–35 μm in diameter (Fig. 4-C, G). They contained only one (rarely two) prominent vacuole 10–15 μm in diameter. Hyaline cytoplasm was observed in these cells, and they occasionally spread but exhibited no obvious movement. Vacuolated cells type 2 (v2-cells) were round or polygonal cells, 30–40 μm in diameter (Fig. 3-B, F), without any pseudopodia. In the Nomarski-image they were filled with several vacuoles 5–8 μm in diameter (Fig. 3-B). In the phase-contrast-image, they were weakly refractile with a relatively dark central area. They were transparent and their contour was indistinct when observed by light microscopy.

Vacuolated cells type 3 (v3-cells) 35–45 μm in diameter contained several large vacuoles which

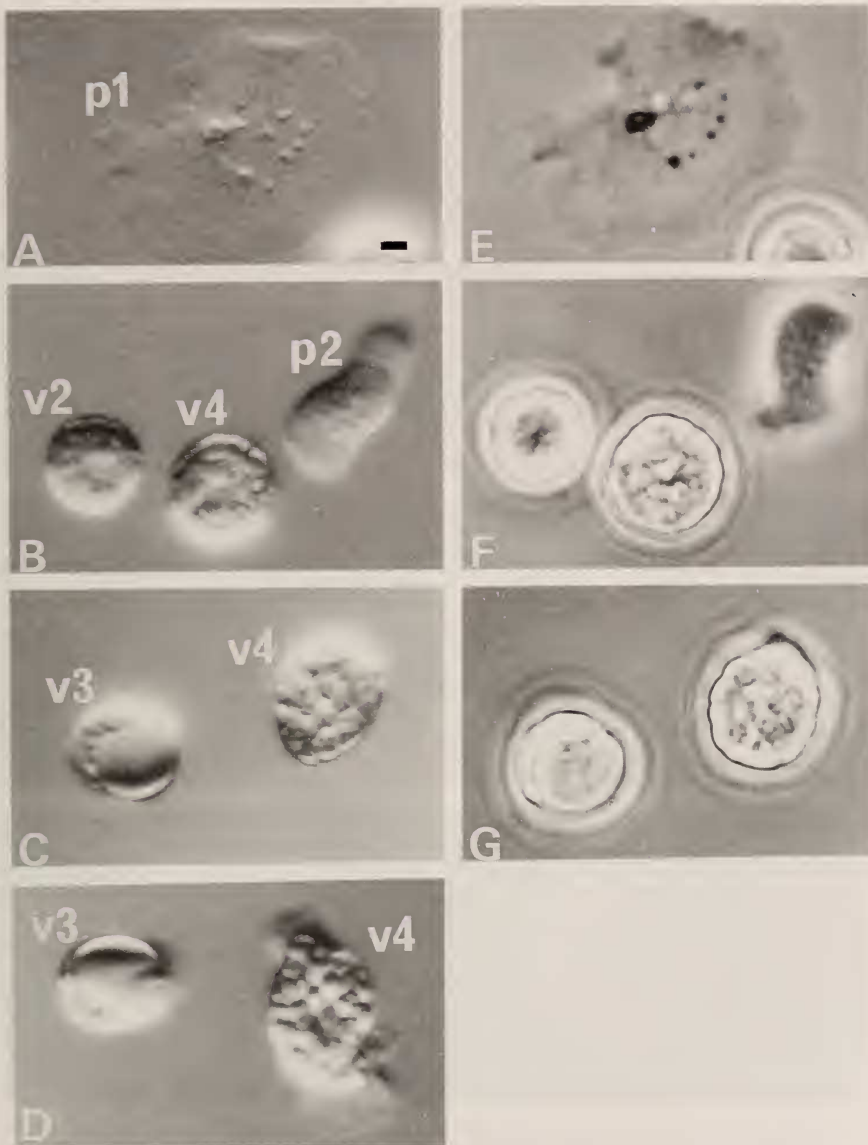


FIG. 3. The five hemocyte types at the condition for adhering cells, in Nomarski-differential-interference-microscopy (A-D) and in phase-contrast-microscopy (E-G). E-G show the same cells as A-C, respectively. D showed the slightly spread type v4 cells when the cover slip was slightly pressed. The scale bar shows 10 μm . p1, p2: phagocyte type 1 and 2; v2, v3, v4: vacuolated cell type 2, 3 and 4.

occupied most of the cytoplasm. In both the phase-contrast- and in the Nomarski-image, they were highly refractile (Figs. 3-C, D, G; 4-C, G). The diameters of their cytoplasmic vacuoles varied from 10 to 30 μm . A hyaline cytoplasm was hardly distinct by phase-contrast microscopy. These cells sometimes moved actively and the refractile

vacuoles changed shape when the entire cell-shape was altered during movement. Vacuolated cells type 4 (v4-cells) 35–50 μm in diameter were also filled with many highly refractile vacuoles both in phase-contrast- and in Nomarski-images (Fig. 3-B, C, D, F, G). The vacuoles closely resembled those of v3-cells while the size of the vacuoles in v4-cells

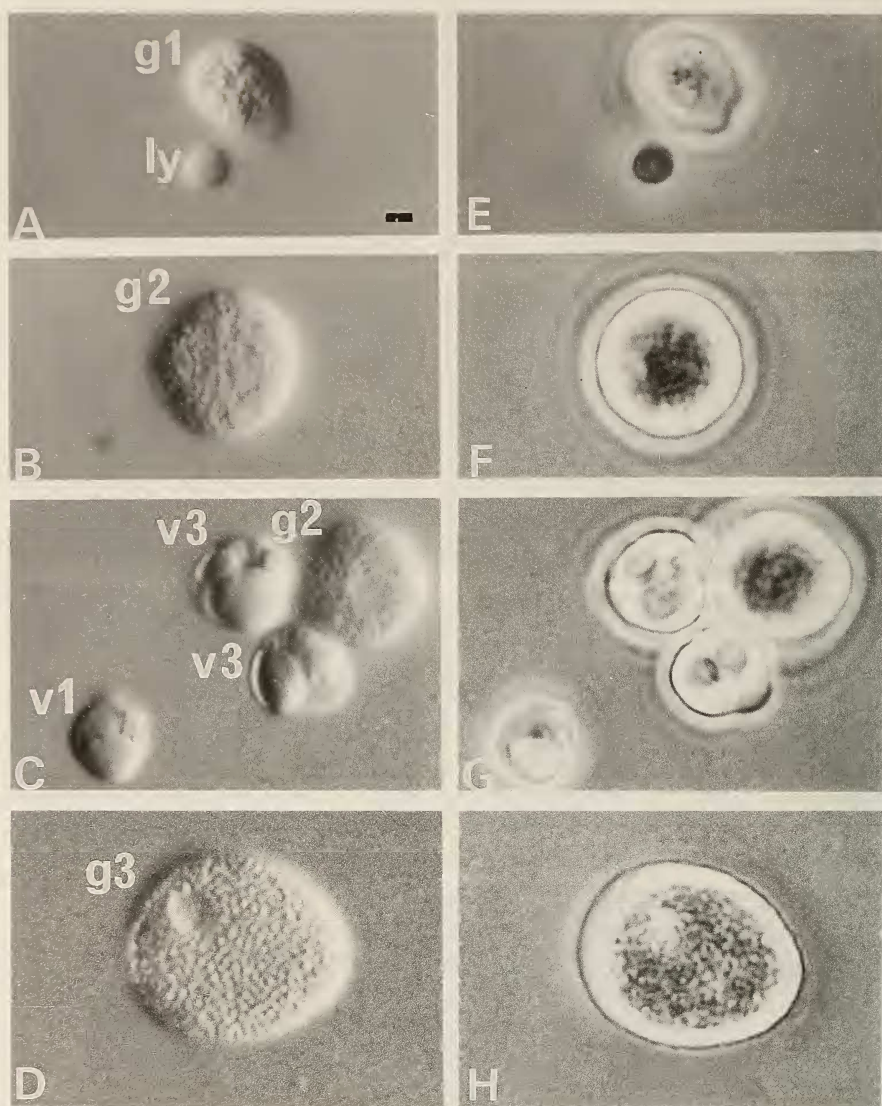


FIG. 4. The six hemocyte types at the condition for adhering cells, in Nomarski-differential-interference microscopy (A-D) and in phase-contrast microscopy (E-H). E-H show the same cells as A-D, respectively. The scale bar shows 10 μm . g1, g2, g3: granular cell type 1, 2 and 3; v1, v3: vacuolated cell type 1 and 3; ly: lymphoid cell.

was relatively uniform within the range of 8–10 μm in diameter (Fig. 3-C, D). V4-cells contained more vacuoles than v3-cells, and v4-cells had higher motility than v3-cells. However, it was sometimes difficult to distinguish v4-cells from v3-cells in the condition for adhering cells. The identification of v3- and v4-cells was easy after we slightly pressed the cover slips which could make the

v4-cells to spread.

Lymphoid cells (ly-cells) were the smallest and the most spherical cells 12–20 μm in diameter (Fig. 4-A, E), appearing as dark spheres in the phase-contrast image. They rarely spread on glass slides, and had no particular characteristics other than their small size.

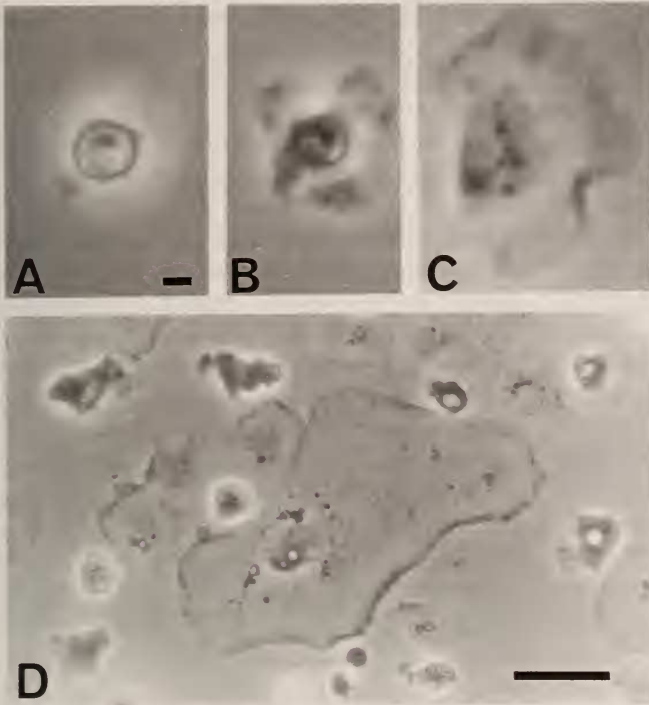


FIG. 5. A, B, C: The change of the shape of phagocyte type 1 with spreading; round (A), slightly spread (B) and completely spread as a sheet (C). The scale bar shows $10\ \mu\text{m}$. D: The syncytium as a large sheet formed by the fusion of spread phagocyte type 1. The scale bar shows $100\ \mu\text{m}$.

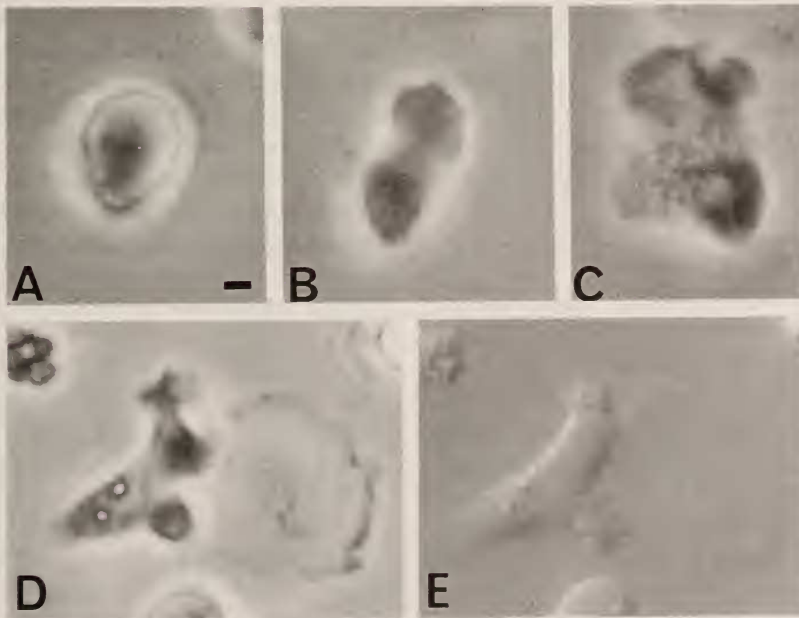


FIG. 6. A, B, C: The change of the shape of a phagocyte type 2 cell with spreading; round (A), slightly long (B) and spread as a dark sheet (C). D, E: Phagocyte type 1 and type 2 spread on a glass slide. Note the difference between two types of cells, in darkness in phase-contrast microscopy (D) and in thickness in Nomarski-differential-interference microscopy (E). The scale bar shows $10\ \mu\text{m}$.

Identification of hemocytes suspended in EGTA-solution

Following the observation of p1- and p2-cells from adhering through spreading on glass slides, we identified both cell types in the non-spreading state in cell suspensions.

In EGTA-solution p1-cells were small cells 20–40 μm in diameter with irregular protrusions (Figs. 1-A; 5), whereas they were small spherical cells with frill-like or spine-like pseudopodia in intact hemolymph. Most of them had small, refractile vacuoles which often disappeared when they spread by adherence.

In EGTA-solution p2-cells were relatively large cells 40–50 μm in diameter, spherical (Fig. 6) or often with irregularly protruding pseudopodia (Fig. 1-A). It was sometimes difficult to distinguish p2-cells from g1-cells in suspension, and the neutral red-staining was useful to identify p2-cells; p2-cells contained cytoplasmic granules which were stained with neutral red while g1-cells were not stained.

The identification of other eight hemocyte types in suspension was possible using the same characteristics as those which adhered (Fig. 1). Other than the ten hemocyte types, we found non-motile cells having cilia-like projections in suspension (Fig. 1-A an arrow). The projections were absorbed into cell-body after adherence when the pH was raised. They were counted as v2-cells after staining them with neutral red.

Other characteristics of hemocytes

Behavior on glass slides in intact hemolymph: Most of p1-cells spread and fused together to form a large syncytium, while they never fused in the EGTA-solution. Many of g1-cells extended long rod-shaped pseudopodia, forming clusters together with the same cell types and at the same time discharging cytoplasmic granules. Sometimes, g2- and g3-cells moved with a small hyaline cytoplasmic cap at their tip or tail without apparent degranulation nor extensive change in shape. Usually, v4-cells spread and were easily distinguished from v3-cells.

Vital staining with neutral red: Within 2–3 min of the staining, v1-, v3- and v4-cells stained rapidly

in red-violet. On the other hand, g3-, p1-, p2- and v2-cells required 5–10 min and they stained orange-red. Neither g1- and g2-cells nor ly-cells stained.

Autonomous fluorescence under the illumination of UV-rays: Intensive and autonomous blue fluorescence was exhibited by v1- and v3-cells. The brightness of the fluorescence increased during first 15–30 sec, then weakened and the color changed into whitish blue. Slightly weak fluorescence was exhibited by v4-cells, but the fluorescence was faster in diminishing than that of v1- and v3-cells. After 2–5 min of illumination, p1- and p2-cells began to exhibit blue fluorescence.

Phagocytosis: Only p1- and p2-cells showed active phagocytosis against SRBCs, engulfing more than 2 and up to 5–7 SRBCs. Little activity of phagocytosis was observed in v4-cells.

Effect of NH_4Cl or NH_4OH : When NH_4Cl (pH 5.0) or NH_4OH (pH 8.0) was added to the hemocyte suspension, the highly refractile cytoplasmic vacuoles of v1-, v3- and v4-cells changed into orange-brown spheres both in EGTA-solution and in intact hemolymph, which we refer to the ammonium-effect (Fig. 7). The ammonium-effect occurred more quickly in the presence of higher concentrations of NH_4Cl or NH_4OH . When the final pH of the hemocyte suspension was shifted within the range of 5.0–8.0 after adding NH_4Cl or NH_4OH , the ammonium-effect occurred similarly throughout that range.

The vacuoles of v1-, v3- and v4-cells also lost the autonomous fluorescence after the ammonium-effect. All hemocytes including p1- and p2-cells did not spread in the presence of NH_4Cl or

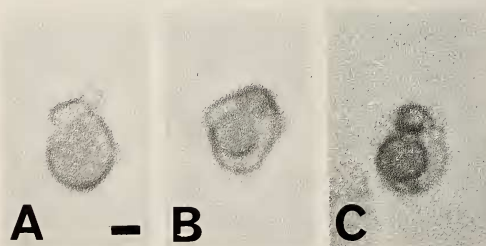


Fig. 7. The vacuolated cell type 3 changing upon the addition of 10 mM NH_4Cl (A; 1 min, B; 5 min and C; 30 min after the addition). The vacuole in A changed into two spherical granules in C. The scale bar shows 10 μm .

TABLE 1. Hemocyte composition of tunicate *Halocynthia roretzi* at different conditions. Each cell type was counted at pH 5.5 (cell suspensions) and at pH 5.9–6.2 (adhering cells).

type of hemocytes	case 1		case 2		case 3	
	Sus* (5.5)	Ad* (6.2)	Sus (5.5)	Ad (5.9)	Sus (5.5)	Ad (6.0)
	(%)	(%)	(%)	(%)	(%)	(%)
p1-cell	34.6	30.3	34.9	33.2	35.4	28.9
p2-cell	7.2	6.6	7.3	6.3	11.6	9.6
g1-cell	13.7	18.8	23.2	18.8	15.0	12.4
g2-cell	1.4	1.4	0.6	1.9	1.9	1.9
g3-cell	0	0	0	0	0	0.3
v1-cell	1.4	1.0	1.0	1.9	0.9	0.9
v2-cell	10.6	10.1	6.0	5.6	10.7	5.3
v3-cell	27.7	24.4	22.2	26.0	21.9	33.5
v4-cell	2.4	3.5	1.6	3.4	0.9	6.2
ly-cell	1.0	3.8	3.2	2.8	1.6	1.6
number of total cell counted	292	287	315	319	319	322

See text for the abbreviation of hemocytes types.

* Sus: cell suspensions (pH)

Ad: adhering cells (pH)

NH₄OH.

Wright's blood stain of fixed hemocytes: After fixation with methanol, we clearly identified only g1- and g2-cells because of their sizes, round shape and frequency. The cytoplasm of both g1- and g2-cells stained dark-blue or violet, definitely indicating that those cells have basophilic cytoplasm. All other cell types appeared to have acidophilic cytoplasm except for a group of small cells whose cell-type was not defined.

The composition of hemocytes (percentage of each cell type in total hemocytes)

The composition of hemocytes analyzed in the condition for adhering cells and in the condition for cell suspension: We added 0.1–0.2 ml of hemocyte suspension (pH 5.5) on glass slides and counted the number of each type first in cell suspensions. Then, we raised the pH of the same hemocyte suspension to 5.8–6.2, added 0.1–0.2 ml on glass slides and then counted adhering cells (Table 1). From three different individuals, the relative proportions of various hemocyte types did not change significantly with changes in conditions.

The composition of hemocytes in fresh-animals and 4–8 weeks-animals: We analyzed the relative proportions of various hemocyte types in five fresh-animals within 2 days after they were collected and in eight 4–8 weeks-animals (Table 2).

TABLE 2. Hemocyte composition of tunicate *Halocynthia roretzi*, in freshly collected animals and in others kept in an aquarium for 4–8 weeks.

type of hemocytes	fresh animals (5 animals)	4–8 weeks-animals (8 animals)
	(%)	(%)
p1-cell	34.1 ± 5.4*	33.8 ± 10.3*
p2-cell	9.4 ± 2.9	9.0 ± 2.1
g1-cell	15.8 ± 3.3	17.7 ± 4.3
g2-cell	1.4 ± 0.4	1.3 ± 0.5
g3-cell	0.2 ± 0.3	0.1 ± 0.1
v1-cell	1.0 ± 0.5	1.1 ± 0.8
v2-cell	5.8 ± 2.8	7.3 ± 2.7
v3-cell	24.8 ± 6.0	23.2 ± 7.2
v4-cell	4.2 ± 1.4	4.6 ± 2.4
ly-cell	3.4 ± 1.3	1.9 ± 2.0

See text for the abbreviation of hemocytes types.

* average ± SD

The analysis was done on adhering cells and the composition of hemocytes in the 4–8 weeks-animals was essentially equal to that in fresh-animals.

DISCUSSION

We propose a standard condition for classifying living hemocytes of *Halocynthia roretzi*, and we have classified them into ten groups (two types of phagocytes, three types of granular cells, four types of vacuolated cells and lymphoid cells). It was also possible to classify hemocytes in suspension, although vital staining was sometimes necessary. The relative proportions of various hemocyte types analyzed as adhering cells and cells in sus-

pension did not differ significantly. Therefore, we considered that a classification in the cell suspensions most nearly correlated with the classification for adhering cells. Thus, adherence of hemocytes on slides did not induce any specific loss of particular hemocyte groups. Because the classification of adhering cells was mainly based on morphological characteristics of living cells, easily observed in light microscopy, it may be useful for investigating hemocyte functions.

We also described the other characteristics, such as autonomous fluorescence or the effect of ammonium on hemocytes (Table 3). Autonomous fluorescence has not been systematically employed in classifying hemocytes, but some investigators have made significant observations (personal com-

TABLE 3. Characteristics of each hemocyte type in tunicate *Halocynthia roretzi*

type of hemocyte	cell size ¹⁾	phagocytosis ²⁾	vital ³⁾ staining	ammonium-effects	Wright's ⁴⁾ blood stain	autonomous ⁵⁾ fluorescence	granules (G) or vacuoles (V)	spreading on ⁶⁾ slide glass (other remark)
p1-cell	80–100 μ m (20–40)	++++	orange	—	a	+	one or some small G	+
p2-cell	60–100 (40–50)	++++	orange	—	a	+	several small G	+
								(active movement)
g1-cell	35–40	—	—	—	b	—	many small G <5 μ m	—
								(rod-shape extension)
g2-cell	45–50	—	—	—	b	—	many small G <5 μ m	—
g3-cell	80	—	orange	+	a	?	many small G <5 μ m	—
								(largest cell)
v1-cell	30–35	—	red-violet	+	a	+	one V 10–15 μ m	—
v2-cell	30–40	—	orange	—	a	—	many V 5–8 μ m	—
v3-cell	35–45	—	red-violet	+	a	+	many large V 10–30 μ m	—
								(refractile)
v4-cell	35–50	+	red-violet	+	a	+	many V 8–10 μ m	—
								(refractile)
ly-cell	12–20	—	—	—	?	—	—	—

See text for the abbreviation of hemocytes types.

¹⁾ Cell-size: adhering cells on glass slides (cell suspension).

²⁾ Only the phagocytic activity against sheep red blood cells (SRBC) was examined.

+: at least some of them could phagocytize SRBC, ++++: they phagocytized many SRBCs actively.

³⁾ Vital staining with neutral red.

⁴⁾ Staining of cytoplasm; a: acidophilic, b: basophilic, ?: uncertain.

⁵⁾ Fluorescence under the UV-illumination; ?: uncertain.

*: Fluorescence gradually appeared after 2–5 min of illumination.

⁶⁾ The ability to spread on slide glasses at the condition for adhering cells.

munication). The effect of ammonium was firstly reported here. We sometimes found many hemocytes with orange-brown granules, similar to those following the effects of ammonium, in animals kept in an aquarium for several months or with dead animals (data not shown). This suggested that the effect of ammonium may occur naturally *in vivo* when animals are poorly maintained. However, we do not know whether or not this reaction has some physiological role that protects the animals.

The classification of hemocytes

Designation: We found no proper designation for hemocytes, although we attempted to use a previous one [8]: large-amoebocyte for p1-cell, macrophage for p2-cell, minute-granular cell for g1-cell, large-basophilic-cell for g2-cell, large-acidophilic-cell for g3-cell, single-granular-cell for v1-cell, morula-cell for v2-cell, vesicular-cell for v3-cell, large-granular-cell for v4-cell, and lymphocyte for ly-cell.

Basically similar cells even from different species are the same. However, we found only little evidence of similarity. So, we attempted to avoid specific designations and to use general terms of hemocyte classification according to Wright [9]. Further refinement of tunicate hemocyte types should continue to be standardized after accumulation of data on functions, the role of the contents of cytoplasmic granules or vacuoles, the ultrastructure and the developmental cell-lineages.

Phagocytic cells: We confirmed phagocytic activity in three groups of hemocytes; p1-, g2- and v4-cells. The clearance of foreign particles or foreign organisms may depend mainly on p1- and p2-cells, because the activity of v4-cells was low. The difference between p1- and p2-cells was distinct in that p1-cells formed a syncytium. Dan-Sohkawa has observed the formation of syncytia by phagocytes of *Halocynthia roretzi* (personal communication), and we consider those phagocytes as p1-cells. Most p1-cells have the granules which disappeared after spreading, suggesting that p1-cells may have another function that of discharging their granular components. P2-cells never fused, and thus p1- and p2-cells belong to a different group which exhibits some phagocytic

activity.

Cells with small granules: There were three groups of hemocytes (g1-3 cells) containing numerous small granules which may be storage organelles for secretory material. P2-cells also had small granular components which stained with neutral red. However, we did not count p2-cells among the cells which contained small granules, since we considered the granular components of p2-cells as phagocytic vesicles or lysosomes rather than as storage sites for secreting substances.

The three types of granular cells were definitely different in cell-sizes, in staining characteristics and behavior. Only g3-cells were stained with neutral red and had acidophilic cytoplasm, while g1- and g2-cells were not stained with neutral red but had apparently basophilic cytoplasm. Only g1-cells aggregated within the same cell type and extended long pseudopodia with active degranulation in normal sea water and intact hemolymph.

Cell containing large vacuoles: There were four hemocyte groups (v1-4 cells) which contained large cytoplasmic vacuoles. Among them, only v2-cells did not exhibit autonomous fluorescence and stained orange-red with neutral red. The other three groups were different not only in cell-size and number of cytoplasmic vacuoles but also in phagocytic activity and volume of hyaline cytoplasm, although their cytoplasmic vacuoles had similar characters.

Correlation to the former classification: Following the reports by Fuke [5] in vital staining, in Wright's blood stain and in cell behavior, we corroborated seven groups according to our classification to those which she classified: p1-cells to vacuolated-cells, p2-cells to fine-granular-cells, g1-cells to minute-granular-cells, g2-cells to large-basophilic-cells, v3-cells to vesicular-cells, v4-cells to large-granular-cells, and ly-cells to lymphocytes.

Our results suggest that orange-cells [5] are not a particular hemocyte group but consists of v1-, v3- and v4-cells under certain sub-optimal conditions. Moreover, we were not able to correlate g3-, v1- and v2-cells to any previously described groups [5]; g3- and v1-cells were both minor groups and might have been neglected. V2-cells accounted for 5-7% of whole hemocytes and could have been considered as vesicular-cells because of their large cyto-

plasmic vacuoles.

The attempt to separate hemocytes by centrifugation in density gradient has been only partially successful using cells of *Halocynthia roretzi*. Azumi *et al.* [3] separated hemocytes into several layers in a density gradient, each of which we could not corroborate with our classification. However, we have confirmed that v3- and v4-cells were in the heaviest group and that p1-cells were in the lightest group in a density gradient-separation using sucrose (unpublished data).

Composition of hemocytes: Despite agreement on the seven groups which constitute the major hemocyte population, the relative proportions of those groups which we observed were different from those of Fuke [5]. We found the percentage of g1-cells to be 12–20%, while the corresponding minute-granular-cells accounted for only less than 5% [5]. In addition, v3-cells accounted for only 20–30% in contrast to the corresponding vesicular-cells which were predominant at 50–65% [5]. The most distinct discrepancy concerned the percentage of p1-cells which resembled the vacuolated-cells. Our results showed that 30–45% of the whole hemocyte fraction was p1-cells, being the largest group. On the other hand the vacuolated-cells accounted for only 4% [5]. We found no seasonal differences from April to October nor variations in non-fed animals. However, those variations, including the quantitative variations of the hemocyte composition remain as subjects for further detailed study.

The question concerning the composition of hemocytes must consider several points. First tunicates do not have a closed circulatory system, isolated from various tissue cavities. Thus certain tissue-specific cells or even hematopoietic cells at various stages of differentiation may occur together and may be possibly collected with hemolymph from certain cavities. Still to be resolved is the composition of hemocytes, and if the variability is due to sampling regions.

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